Enhancement of ectopic osteoid formation following the dual release of bone morphogenetic protein 2 and Wnt1 inducible signaling pathway protein 1 from gelatin sponges.

AUTHOR(S):

Kohara, Hiroshi; Tabata, Yasuhiko

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Abbreviated title: Ectopic Osteogenesis by Dual Release of BMP2 and WISP1

Hiroshi Kohara, Ph.D.[a], and Yasuhiko Tabata, Ph.D., D.Med.Sci., D.Pharm.[a]

[a]*Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University*

Correspondence: Yasuhiko Tabata

Department of Biomaterials, Field of Tissue Engineering,

Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho Shogoin,

Sakyo-ku, Kyoto 606-8507, Japan

Phone: +81-75-751-4128

Fax: +81-75-751-4646

Email: yasuhiko@frontier.kyoto-u.ac.jp

Abstract

Bone morphogenetic protein (BMP) 2-incorporated gelatin sponge is effective for *in vivo* osteoinduction. However, the modeling capacity of bone decreases with age. As a trial to stimulate effective bone formation for animals with decreased osteogenic potential, Wnt1 inducible signaling pathway protein (WISP) 1, an osteoblastic regulator, was combined with gelatin sponge incorporating BMP2. *Osteopontin* (*Opn*) gene expression was increased *in vitro* for mouse bone marrow stromal cells (BMSC) cultured in gelatin sponges incorporating BMP2 and WISP1 compared with those incorporating BMP2 or WISP1 alone. *In vivo* synergistic effect of BMP2 and WISP1 on the ectopic osteoid formation was observed when gelatin sponges incorporating BMP2 and WISP1 were implanted subcutaneously into middle-aged mice with decreased bone formation potential. It is concluded that the scaffold incorporating multiple osteoinductive agents could be effective in inducing bone formation in those with age-related decreased potential of bone formation.

Keywords

Controlled release, BMP2, WISP1, Bone formation, Age-related change

1. Introduction

It is well known that bone formation is impaired with aging [1, 2]. The bone is composed of extracellular matrix proteins, including type I collagen, stiffened by the crystals of calcium hydroxyapatite, and bone loses its matrix and mineral composition with advancing age [1, 3]. At the cellular level, bone formation is a function of the osteoblast cell lineage, whereas bone resorption is regulated by cells of the osteoclast linage [4, 5]. It has been also reported that aging is accompanied by the alteration of the relationship between osteoblasts and osteoclasts [6, 7]. In the rat model of ectopic bone formation, bone tissue induced ectopically by the subcutaneous implantation of BMP2 into rat back skin was reduced with increasing age [8].

The bone morphogenetic protein (BMP) 2 incorporated in porous biodegradable scaffold is useful for bone tissue engineering. BMP subfamily within the transforming growth factor- (TGF-) superfamily is a group of proteins with osteoinductive activity [9, 10]. Among the BMP subfamilies, BMP2 was originally cloned from the bovine bone [11]. It has been also shown that recombinant human BMP2 induces ectopic bone formation in rats [12]. The advantage of BMP2 in bone tissue engineering is to the clinical application for bone regeneration at the bone defect [13]. It has been demonstrated that a biodegradable hydrogel of gelatin is used for the

controlled release of BMP2 [14]. Generally, the direct injection of growth factor solution into the site to be regenerated is not therapeutically effective. This is because the water-soluble factors are rapidly excreted from the injected site and often digested or deactivated. BMP2 was retained in the gelatin hydrogel *in vivo* for more than 1 month and long-termed BMP2 release was achieved [14]. BMP2-incorporated three-dimensional porous structure of gelatin hydrogel, what has been referred to as "gelatin sponges", is effective for osteoinduction both *in vitro* and *in vivo* [15, 16].

As one of the trials to stimulate effective bone formation in the patients with decreased osteogenic potential, bone tissue engineering approach to combine scaffold with multiple osteoinductive agents is worth examining. Wnt signaling pathway is predicted to control bone mass because of the increased bone mass in human with mutations in the Wnt receptor low density lipoprotein receptor-related protein (LRP) 5/6 [17, 18]. Wnt1 inducible signaling pathway protein 1 (WISP1; also known as CCN4) is a member of CCN family protein, originally cloned as the target gene of the Wnt1/Frizzled pathway in the breast cancer cells [19]. It has been shown that recombinant WISP1 promotes proliferation and osteogenic differentiation of human bone marrow stromal cells (BMSC) *in vitro* [20]. Interestingly, human BMSC co-transduced WISP1 and BMP2 had significantly greater ectopic bone formation than

those transduced with BMP2 alone when implanted into the back of mice [20].

In this study, both BMP2 and WISP1 were incorporated into gelatin sponges, and the *in vitro* osteogenic activities of gelatin sponges incorporating BMP2 and/or WISP1 were investigated. Ectopic bone formation *in vivo* was investigated by the subcutaneous implantation of gelatin sponges incorporating BMP2 and/or WISP1 into mice of a middle age, in which the bone formation activity declines, and compared with that of sponges incorporating BMP2 or WISP1.

2. Materials and Methods

2.1. Materials

A gelatin sample with an isoelectric point (IEP) of 9.0 was prepared through an acidic process of porcine skin collagen type I (Nitta Gelatin Co., Osaka, Japan). β-tricalcium phosphate (β-TCP) granules (2 μm in average diameter) were obtained from Taihei Chemical Industries, Nara, Japan. $Na^{125}I$ (740MBq/ml in 0.1N NaOH aqueous solution) was purchased from Du Pont NEN Research Products (Wilmington, MA, USA). Glutaraldehyde (GA), glycine, and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification. The monoclonal antibodies 30-F11 (anti-CD45) and D7 (anti-Sca-1) were from eBioscience,

Inc. (San Diego, CA, USA). All primers (Table 1), SuperScript VILO and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, USA). RNeasy Plus Mini Kit was from Qiagen Inc. (Valencia, CA). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Fetal bovine serums (FBS) were purchased from Hyclone (Logan, UT, USA). Propidium iodide was obtained from Dojindo Laboratories (Kumamoto, Japan). 2.4G2 hybridoma was purchased from American Type Culture Collection (Rockville, MD, USA). WISP1 was purchased from PeproTech (Rocky Hill, NJ, USA) .

2.2. Mice

C57BL/6NCrSlc female mice were purchased at 4-12 weeks old from Shimizu Laboratory Supplies Co. (Kyoto, Japan) and were used between 6 and 38 weeks old. All animal experimentation was conducted in accordance with the guidance of the Institute for Frontier Medical Sciences, Kyoto University.

2.3. Preparation of gelatin sponges with β-TCP

Gelatin sponges incorporating β-TCP were prepared as described previously [15]. Briefly, 4.29 wt% aqueous solution of gelatin (70 ml) was mixed at 5000 rpm at 37◦C

for 3 min by using a homogenizer (ED-12, Nihonseiki Co., Tokyo, Japan). After addition of and 10 wt% of β-TCP and 2.17 wt% of glutaraldehyde aqueous solution (30 ml), the mixed solution was further mixed for 15 s by the homogenizer. The resulting solution was cast into a polypropylene dish of 138 x 138 mm^2 and 5 mm depth, followed by leaving at 4℃ for 12 hr for gelatin crosslinking. Then, the cross-linked gelatin hydrogels with β-TCP were placed into 100 mM of aqueous glycine solution at 37◦C for 1 hr to block the residual aldehyde groups of glutaraldehyde. Following complete washing with double distilled water (DDW), the hydrogels were freeze-dried and cut into disks (5 mm diameter).

2.4. In vitro release study of BMP2 and WISP1 from gelatin sponges

BMP2 and WISP1 were radioiodinated through the conventional chloramine-T method as previously described [21]. Briefly, 5 ml of $Na^{125}I$ was added to 50 μl of 5 mg/ml BMP2 or 1 mg/ml WISP1 solution in 0.5 M potassium phosphate buffer (pH 7.5) containing 0.5 M NaCl. Then, 0.2 mg/ml chloramine-T in the same buffer (100 μl) was added to each solution mixture. After agitation at room temperature for 2 min, 100 μl of phosphate-buffered saline (PBS) solution (pH 7.5) containing 0.4 mg sodium metabisulfate was added to the reaction solution to stop the radioiodination. The

reaction mixtures were passed through PD-10 columns to remove the uncoupled, free ^{125}I molecules from the ^{125}I-labeled BMP2 and WISP1. A PBS solution of ^{125}I-labeled BMP2 and/or WISP1 (20 μl) was dropped onto the freeze-dried sponges of gelatin (5 mm diameter), followed by leaving at 25 °C for 3 h to obtain hydrogel sponges incorporating ^{125}I-labeled BMP2, WISP1, or both. For the *in vitro* release test, hydrogels incorporating ^{125}I-labeled cytokines were agitated at 37 °C in 1 ml of PBS. The supernatant was removed 1, 2, 4, 6, 12, and 24 h later and replaced with the same volume of fresh PBS. The radioactivity of each supernatant was measured on a gamma counter (ARC-301B, Aloka, Tokyo, Japan) to evaluate the time profile of cytokines release (n = 3 at each time point).

2.5. In vitro culture of BMSC in gelatin sponges

To isolate BMSC, fresh bone marrow cells were harvested from femurs and tibias of the euthanized mice (7 wk old), and were suspended in DMEM supplemented with 2% FBS and penicillin/streptomycin. The red blood cells were hemolyzed in BD Pharm Lyse Lysing Buffer (BD Bioscience, Rockville, MD, USA) under the manufacturer's guidance. The cell suspensions were filtered through a cell strainer (BD Bioscience) to remove debris. The filtrates were pelleted by centrifugation for 5 min at 4 ◦C. The bone

marrow cells were resuspended at 5 x 10^6 cells/ml in DMEM supplemented with 10% FBS and penicillin/streptomycin, and plated onto 100-mm culture dish. The adherent cells were passaged two times before use.

The BMSC at passage two were incubated with 1 ml of the 0.25% trypsin/EDTA solution for 5 min at 37◦C, until the cells detach from the dishes. The cells were washed by centrifugation, and seeded onto the gelatin sponges in which BMP2 and/or Wips1 were incorporated as described above. BMSC were resuspended in DMEM supplemented with 10% FBS, 50 μg/ml ascorbic acid-2- phosphate , 10 mM β-glycerophosphate, 10^{-8} M dexamethasone. The cell suspensions were seeded into the gelatin sponges by an agitated seeding method, by which cells were seeded homogeneously throughout 3-dimensional porous scaffolds [15].

2.6. Quantitative, real-time polymerase chain reaction with reverse transcription (qRT-PCR)

The total RNA was extracted from BMSC cultured for 7 days in gelatin sponges incorporating BMP2 and/or Wips1 by using RNeasy Kit according to the manufacturers' instructions. Reverse transcription reaction was performed with SuperScript VILO. Real-time PCR was performed on a Prism 7500 real-time PCR thermal cycler (Applied

Biosystems, Foster City, CA, USA) from 10 ng of cDNA in a total volume of 25 ml containing Power SYBR Green PCR Master Mix and 10 mM of each primer (Table 1). The reaction mixture was incubated for the initial denaturation at 95°C for 10 min, followed by 40 PCR cycles. Each cycle consisted of the following three steps; 94°C for 15 s, 57°C for 15 s and 72°C for 1 min. Each mRNA level was normalized by the expression level of 18S ribosomal RNA as an internal control (n = 3 at each treatment combination).

2.7. In vivo osteogenic ectopic assay

Gelatin hydrogels incorporating BMP2 and/or WISP1 as described above were implanted into the back subcutis of mice at various ages (6 mice/experimental group). The BMP2-free, WISP1-free empty gelatin hydrogel were used as controls. The skin tissue including the hydrogel-implanted or injected site was taken out for following flow cytometric and histological assays 10 days later.

2.8. Flow cytometric analysis

Gelatin implants harvested were digested as described previously (Matsuzaki Stem cells). Briefly, Gelatin implants were harvested and digested in a solution containing

400 U/mL collagenase D (Roche, Basel, Switzerland) for 40 minutes at 37°C. The resulting single-cell suspensions were blocked by anti-CD16/32 antibody, washed and stained with monoclonal antibodies and its secondary reagents in PBS containing 2 vol% FBS and 0.1 vol% sodium azide. Propidium iodide was used to distinguish dead cells from viable cells. The immunostained cells were analyzed on FACSCanto II flow cytometer. Analysis was performed by BD FACSDiva software (BD Bioscience) and FLOWJO software (Tree Star, San Carlos, CA).

2.9. Histological evaluation of bone tissue ectopically induced by gelatin implants

Gelatin implants were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS. Fixed samples were embedded in OCT medium (Sakura) and frozen in liquid nitrogen. Sections of undecalcified samples were generated via Kawamoto's film method (Cryofilm transfer kit; FINETEC). The 7 mm thick cryostat sections were and stained by hematoxylin and eosin (H&E) to view on an optical microscope (AX-80, OLYMPUS, Japan).

2.10. Colony forming unit-fibroblast (CFU-F) assay

CFU-F assay was performed as described previously (Benedetto AD, J cell science,

2010). In brief, single cell suspensions from gelatin hydrogel implants were suspended in αMEM supplemented with 2% FBS, penicillin and streptomycin using a 18-gauge needle, and then filtered through a 70 μm cell strainer (Falcon) to remove debris. The resulting bone marrow cell suspensions were rinsed in PBS, resuspended in MSC Basal Medium (Stem Cells Technologies) supplemented with MSC Stimulatory Supplement (Stem Cells Technologies) and plated for determination of the CFU-F. Non-adherent cells were removed by changing medium. After 2 weeks of culture, the cells were fixed in ethanol and stained by using Hemacolour Staining Kits (Merck) under the manufacturer's guidance. Colonies containing more than 16 cells were counted (n = 4 at each treatment combination).

2.11. Statistic analysis

All the results were expressed as the mean ± standard deviation (SD). Significant analysis between the experiment groups was done based on the one-way ANOVA, and the difference was considered to be significant at $p < 0.05$.

3. Results

3.1. Release profiles of BMP2 and WISP1 from gelatin sponges with β-TCP

Figure 1 shows *in vitro* release profiles of ^{125}I-labeled BMP2 and/or WISP1 from the gelatin sponges. The radioactivity remaining in the gelatin sponges decreased with the incubation time, and reached a plateau level 4 hr after incubation. A higher amount of BMP2 (>90%) was retained in gelatin sponges 24 hr after incubation, in consistent with previous reports [14]. Although the lower amount of WISP1 (approximately 56%) was retained in gelatin sponges after 24 hr of incubation, it is highly expected that the amount is large enough to induce additive or synergistic effects on ectopic bone formation. The decrement patterns were not influenced by the co-incorporation of BMP2 and WISP1.

3.2. In vitro activities of gelatin sponges incorporating BMP2 and/or WISP1

Figure 2 shows qRT-PCR results of mouse BMSC cultured for 7 days in gelatin sponges incorporating BMP2 and/or WISP1. Gene expression of *runt related transcription factor* (*Runx*) *2*/core-binding factor alpha (*Cbfa*) *1*, a master regulator of osteoblast-specific gene expression, was not significantly affected by co-incorporation of BMP2 nor WISP1 at this time point (Figure 2A). In contrast, the expression of

osteopontin (*Opn*)/*secreted phosphoprotein* (*Spp*) *1*, secreted protein of osteoblasts, increased in BMSC cultured in gelatin sponges incorporating BMP2 and WISP1 compared with other incorporation conditions (Figure 2B).

3.3. Time course of cell infiltration into the gelatin sponge implants

The results of *in vitro* culture prompted us to evaluate *in vivo* osteogenic activities of gelatin sponges incorporating BMP2 and/or WISP1. Figure 3 shows the temporal changes of cell infiltration into the gelatin sponges incorporating BMP2. Infiltration of $CD45^+$ hematopoietic cells reached a small peak at the earliest phase, and increased at 10 days after implantation. In contrast to the $CD45^+$ cells, infiltration of $CD45^-Sca\text{-}1^+$ endothelial and stromal lineage cells [22-24] were detected only 10 days after implantation. Based on these data, we focused our analysis on the ectopic bone formation and cells recruitment into the site of bone formation at 10 days after implantation.

3.4. Age-dependent changes in ectopic bone formation

Gelatin sponges incorporating BMP2 were implanted subcutaneously into mice with various ages, ranging from young to middle-aged. Figure 4 shows the histological

sections of subcutaneous tissues 10 days after implantation. The histological analysis revealed that every BMP2-incorporated gelatin sponge induced osteoid formation homogeneously around the sponges (Figure 4A). The porous structure of gelatin sponge was surrounded by the ectopically formed osteoid tissue. Younger mice had a significantly greater bone formation than older mice (Figures 4A and 4B). Ade-dependent decrease of bone area was in good accordance with the previous report on rats [8].

Figure 5 shows the results of CFU-F assay initiated with the cells from gelatin sponges incorporating BMP2 and implanted subcutaneously. After 14 days in culture, fibroblast-like colonies containing 10-200 cells were observed (Figure 5A). The number of colonies decreased with increasing age (Figure 5B). It is in good correspondence with the result of histological section.

3.5. Effect of dual release of BMP2 and WISP1

From the age-stratified analysis, we chose middle–aged mice, in which the ability to induce ectopic bone formation is decreased, to evaluate the effect of dual release of BMP2 and WISP1. Figures 6A and 6B shows the histological analysis results of gelatin sponges incorporating BMP2 and/or WISP1. Ectopic osteoid formation was not

observed by the implantation of gelatin sponges incorporating only WISP1 (Figures 6A and 6B). The areas of osteoid tissues were significantly increased in the site of implantation of gelatin sponges incorporating both BMP2 and WISP1, compared with that incorporating only BMP2 (Figures 6A and 6B). On the other hand, we observed an increase in the number of fibroblastic colonies (CFU-Fs) to lesser extent than observed in histological analysis (Figure 6C).

4. Discussion

Our results demonstrated that the dual release of BMP2 and WISP1 has potential to improve ectopic bone formation in mice with decreased ability to induce ectopic bone formation. The gelatin sponge incorporating β-TCP was prepared as a release carrier for BMP2 and/or WISP1. *In vitro*, *Opn* expression increased for BMSC cultured with gelatin sponges incorporating BMP2 and WISP1 compared with that of other groups. Moreover, the ectopic osteoid formation was enhanced by the implantation of gelatin sponges incorporating BMP2 and WISP1 in middle-aged mice.

We used gelatin hydrogel with porous structure incorporating β-TCP, i.e. gelatin sponge, as the release carrier. Biosafety of biodegradable gelatin has been proven through its long clinical usage as a plasma expander, in surgical biomaterials and

as an ingredient in drugs [25]. Our previous study demonstrates that gelatin hydrogel incorporating BMP2 have the *in vivo* osteoinductive activity [14]. The porous structure of gelatin hydrogels allowed for cell infiltration, and the incorporation of β-TCP improvee the mechanical strength of porous gelatin sponges [15, 16]. In the present study, homogeneous osteoid formation around the implant was observed by using gelatin sponges incorporating BMP2 in all ages of mice tested (6 – 38 wk). Thus we believe that βTCP-reinforced gelatin sponges incorporating BMP2 is one of the useful tools to induce ectopic bone formation. In this system, the correlation between the gelatin degradation and BMP-2 release was observed, which indicates that BMP2 is released from the gelatin sponge based on the *in vivo* degradation of gelatin [15, 16]. In the *in vitro* release study, severe burst release of WISP1 from the gelatin sponge was not observed. It is conceivable that WISP1 remaining in gelatin hydrogel was released as the result of the hydrogel degradation *in vivo*.

In this study, the basic-type gelatin (isoelectric point [IEP] = 9.0) was used as the carrier material for BMP2 and/or WISP1 release. The electric nature of gelatin can be changed by the collagen processing method, and the acidic process yields basic gelatin with an IEP of 9.0 [25]. The basic gelatin is preferable as the carrier for acidic proteins release, while the acidic gelatin is applicable to the sustained release of basic

proteins. However, WISP1 has an IEP of 9.2 and is slightly positively charged at the physiological pH [26]. Relatively lower amount of WISP1 retained in *in vitro* release study may be explained by the electric nature of WISP1 and gelatin. For better osteoinduction, combination of BMP2-incorporated basic gelatin and WISP1-incorporated acidic gelatin may improve WISP1 release profile and consequently the synergistic effect of BMP2 and WISP1.

The CCN family consists of six distinct proteins (i.e. cysteine-rich 61; Cyr61/CCN1, CTGF/CCN2, nephroblastoma overexpressed; Nov/CCN3, WISP1, 2, and 3; and WISP1–3/CCN4–6). It has been demonstrated that CCN family proteins contribute to biological processes in development, tissue repair, and tumor suppression, but their exact functions are still unspecified [27]. It has been reported that WISP1 enhanced the ability of BMP2 to direct BMSC toward osteogenic differentiation *in vivo* and *in vitro*. Human BMSC transduced with WISP1- and BMP2-adenovirus in a combinational fashion expressed significantly high levels of osteogenic markers including *Opn in vitro*, and induced ectopic bone formation when implanted under the skin of immunocompromised mice [20]. Our results of *Opn* gene expression and ectopic osteoid formation are comparable to this report. It has been shown that WISP1 bound directly to BMP2 and that WISP1 increased BMP2 binding to BMSC [20]. Taken

together, it is likely that WISP1 enhance the effect of BMP2-induced osteoid formation by mesenchymal cells recruited into the site of ectopic osteoid formation.

It is well known that, during osteogenesis, Runx2 works as a multifunctional transcription factor that controls the expression of various extracellular matrix protein genes including *Opn*, *integrin binding sialoprotein* (*IBSP*), and *collagen type I* [28]. At the time point checked in this study, there was no significant effect on *Runx2* expression for BMSC cultured in gelatin sponges incorporating BMP2 and WISP1 compared with that in other sponges, even though the *Opn* expression increased. Since Runx2 works at an early phase of osteogenesis, it is necessary to understand the timecourse of changes of *Runx2* and *Opn* expression for further elucidation of the mechanism.

In the *in vivo* experiments, the cellular level analysis of ectopic osteoid formation was performed. It has been shown that bone marrow-derived cells that have osteogenic potential, named as marrow-derived osteogenic progenitor cells (MOPC), are recruited from the blood circulation into the implantation site of BMP-2 incorporated carrier material [29-31]. In this study, Sca-1 was used as a marker for endothelial/stromal lineage cells. It has been reported that the anti-Sca-1 antibody detects some endothelial cells and non-hematopoietic, non-endothelial stromal cells in the bone marrow [22, 24]. It is possible that Sca-1 marks endothelial/stromal lineage

cells in the ectopic site of osteogenesis just as in the bone marrow. The primitive mesenchymal cells in non-hematopoietic tissues were initially referred to as CFU-F, because they adhered to culture dishes and formed fibroblast-like colonies [32, 33]. Primary CFU-F assay has been recently used to quantitate the numbers of mesenchymal stromal cells (MSC) in the bone marrow [23]. CFU-F colonies were identified in the cells isolated from the subcutaneous tissue implanted with gelatin sponges incorporating BMP2, indicating that CFU-Fs may be an index of a primitive mesenchymal cells migrated into the site of ectopic bone formation. In this study, the number of CFU-Fs decreased with age. It is uncertain whether such a decrease in the number of CFU-Fs is cause or result of age-dependent decrease in the activity of ectopic bone formation. Even so it is possible that further research on the biological contribution of cells infiltrated into the site of bone formation can be feedbacked to the design and fabrication of novel scaffolds for bone tissue engineering.

There are several treatments for bone defects which are caused by resection of malignant bone tumors, traumatic injury, and congenital anomaly; implantation of bioceramics or autogenous and allogenous bone grafts. Although such treatments are extremely popular, there are some risks of the shortage of donor supply, the persistence of pain, the nerve damage, fracture, and cosmetic disability at the donor site with

autografts, disease transmission and immunological reaction with allografts [34, 35]. In addition, the potential of bone regeneration decreases with age in human. Bone tissue engineering may thus be another choice for the treatment of bone defects to resolve the problems [36-38]. A combination of multiple osteoinductive agents and biodegradable scaffolds may provide an appropriate osteoinductive environment for osteogenic cells even in the patients with decreased osteogenic potential. In the present work, as one initial trial, an ectopic bone formation model was applied to evaluate the effect of BMP2 and WISP1 dual release on the bone regeneration. Further investigation is required to determine whether or not the dual release approach is feasible with different animal models considering the clinical treatment of human bone defects.

Conclusion

We investigated the potential of gelatin sponges incorporating BMP2 and WISP1 in the induction of bone formation for middle-aged mice with a decreased osteogenic potential. Ectopic osteoid formation was synergistically enhanced by the combined WISP1 and BMP2 incorporated in gelatin sponges. This indicates that WISP1 enhanced the effect of BMP2-induced osteogenesis through the migration of mesenchymal cells into the site of osteoid formation. It is, therefore, concluded that combination of biodegradable cell

scaffold and dual release of osteogenic agents, such as BMP2 and WISP1, have therapeutic potential for patients with decreased ability of bone formation.

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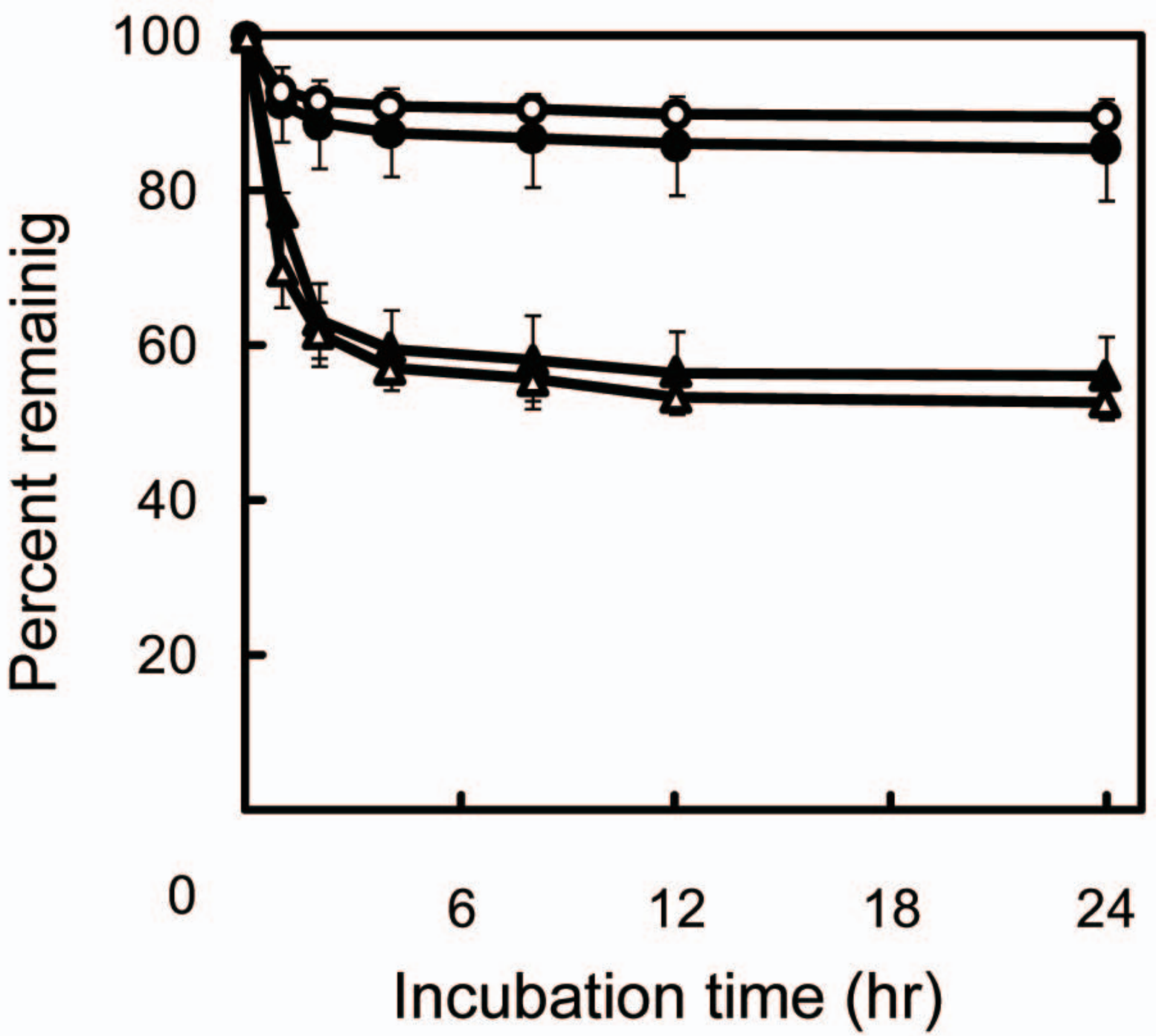

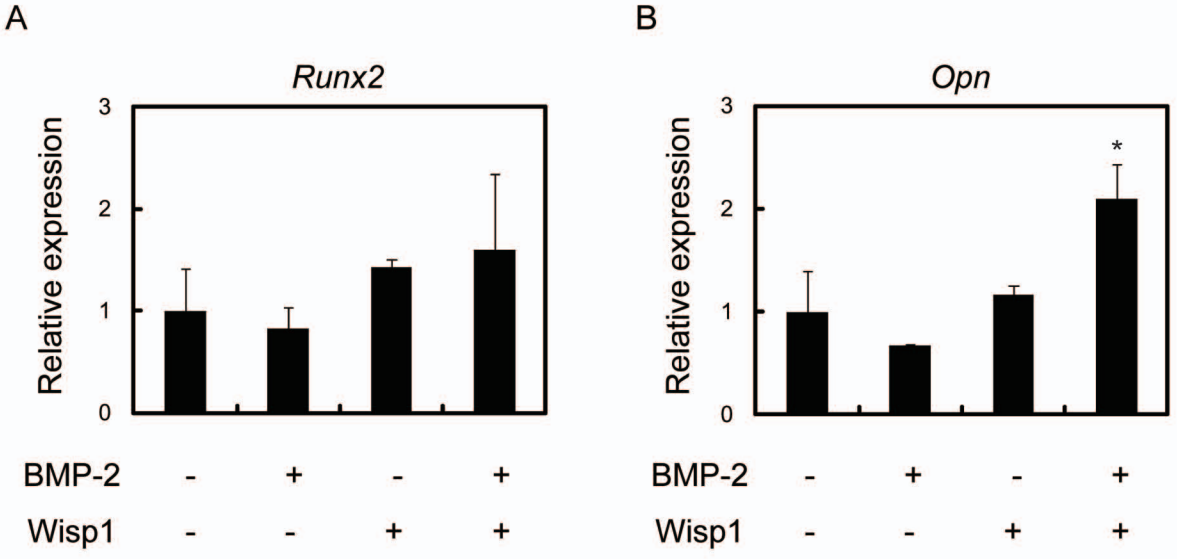

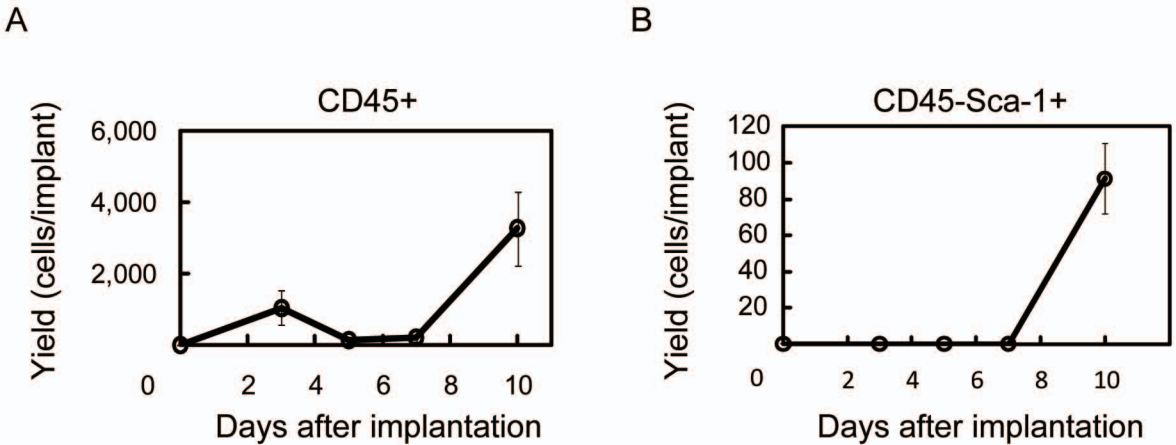

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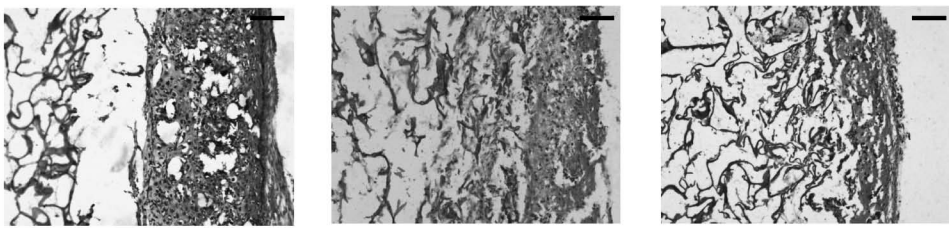

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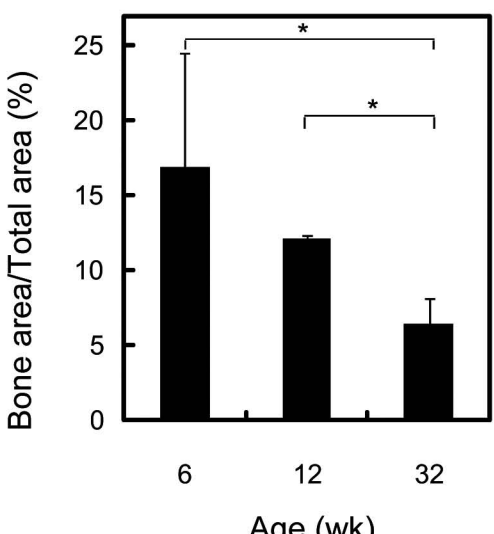

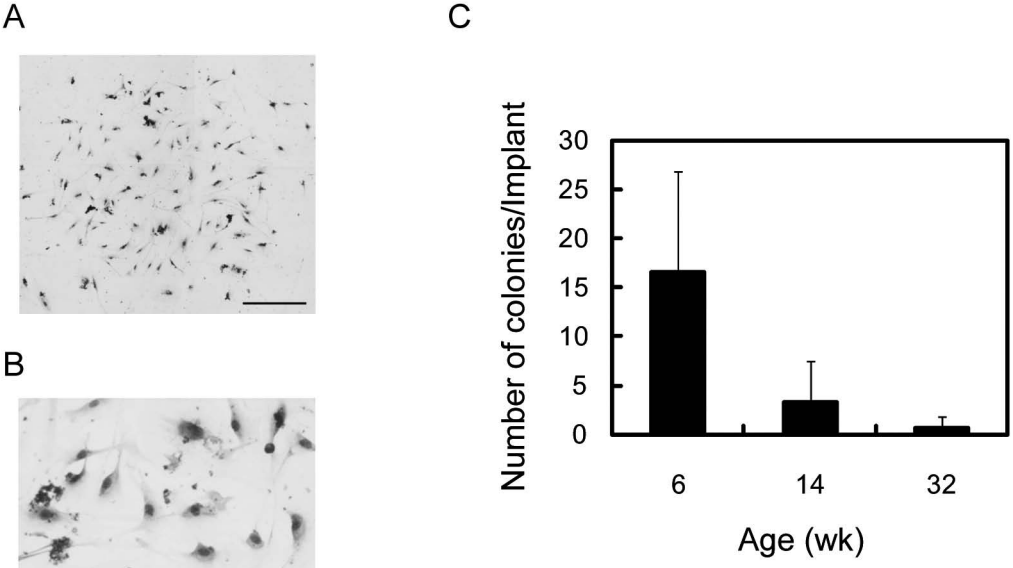

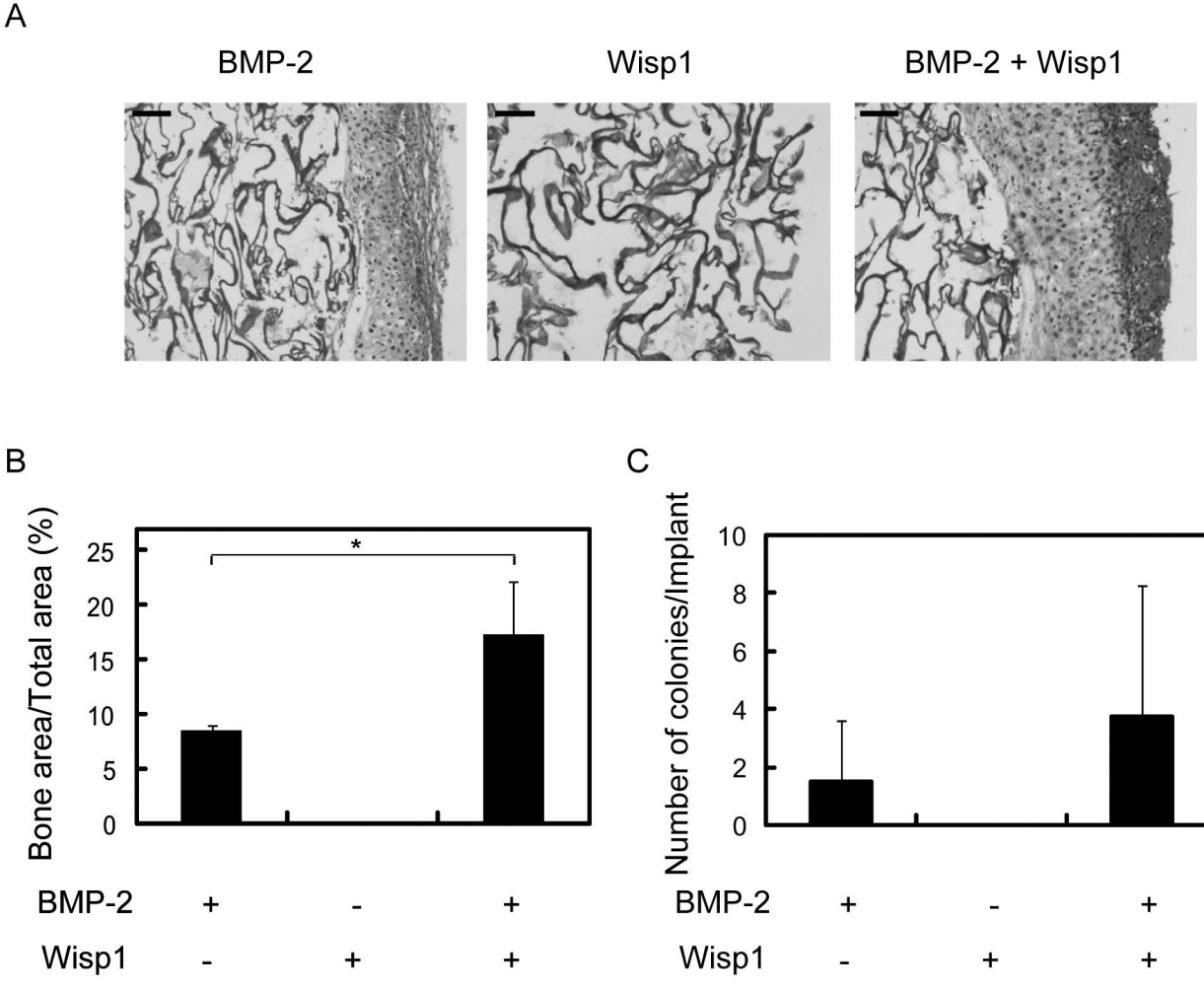

Table 1 Primer sequences used for qRT-PCR

Gene	Primer sequence
Runx2	(S) CCAAGTAGCCAGGTTCAACG
	(AS) TGGGGAGGATTTGTGAAGAC
Opn	(S) GCTTGGCTTATGGACTGAGG
	(AS) AGGTCCTCATCTGTGGCATC
18S	(S) ACTCAACACGGGAAACCTCA
	(AS) AACCAGACAAATCGCTCCAC